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## Import and processing of heart mitochondrial cyclophilin D

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Cyclophilins are a family of cyclosporin-A-binding proteins which catalyse rotation about prolyl peptide bonds. A mitochondrial isoform in mammalian cells, cyclophilin D, is a component of the permeability transition pore that is formed by the adenine nucleotide translocase and the voltage-dependent anion channel at contact sites between the inner and outer membrane. This study investigated the submitochondrial location of cyclophilin D by following the fate of radiolabelled protein following import. Precursor [ $^{35}$ S]cyclophilin D was expressed *in vitro* from a PCR-generated cDNA. The precursor was imported by rat heart mitochondria and processed in a single step to a 21-kDa protein that was identical (SDS/PAGE) to an *in vitro* expressed mature protein and a cyclophilin D purified from rat heart mitochondria. No further modification of the mature protein could be demonstrated. Fractionation of mitochondria following import established that cyclophilin D locates only to the matrix. It is concluded that cyclophilin D-binding to the permeability transition pore must occur at the inner face of the mitochondrial inner membrane.

**Keywords:** cyclophilins; import; mitochondria; polymerase chain reaction.

Cyclophilins form a ubiquitous family of proteins characterized by their ability to catalyse the *cis-trans* isomerization of peptidyl-prolyl (Xaa-Pro) bonds [1,2]. They are also the primary binding targets of the immunosuppressive agent, cyclosporin A (CSA) [3]. Mitochondrial isoforms of cyclophilin, termed cyclophilin D (CyP-D), have been identified in *Neurospora* [4], yeast [5], human [6] and rat [7,8]. These contain an N-terminal targeting sequence that directs the translated proteins to the mitochondria. One function of cyclophilins appears to be the catalysis of nascent protein folding [9]. This is indicated for yeast mitochondrial cyclophilins through the inhibition of protein folding by CSA [10,11].

The observation that CSA potently inhibits opening of the permeability (PT) pore [12] has also implicated CyP-D in pore regulation. The PT pore forms large nonselective pores within the inner mitochondrial membrane [13,14]. Pore opening leads to losses of matrix volume control and energy transduction. The physiological role of the pore has not been established, although it may be involved in necrotic death following ischaemia/reperfusion [12,15–18] and apoptotic cell death under a range of physiological stimuli [19].

Further evidence that CyP-D regulates the PT pore is based on its physical association with pore components. The primary components of the pore are the adenine nucleotide translocase (ANT, inner mitochondrial membrane) [20] and the voltage-dependent anion channel (VDAC, outer mitochondrial membrane) [21]. CyP-D has been copurified with both following the

isolation of intact contact sites from mitochondria [22,23]. Reconstitution experiments show that these contact sites can insert into liposomes and form PT pore-like channels. Recently, we have demonstrated direct binding of recombinant CyP-D to ANT/VDAC complexes [24]. These complexes also form channels within proteoliposomes that show similar properties to the PT pore itself. In parallel work, Woodfield, *et al.* [25] demonstrated binding of CyP-D to ANT alone.

Purification of mitochondrial cyclophilin from rat leads to the recovery of two proteins of 19 kDa and 18 kDa [7] or 21 kDa and 18 kDa [8]. Either, or both, could regulate the PT pore. Protein sequencing [7,26] demonstrated that the larger forms were homologous to human CyP-D (or cyclophilin 3) [6]. The N-terminal sequence of the short form was identical to that located within 10 amino acids of the N-terminal of the larger form [7], implying that the smaller form is derived by N-terminal truncation of the larger form. Such two-step processing is typical of many mitochondrial proteins [27]. Subsequent cloning of the cDNA for rat CyP-D demonstrated that it was nuclear derived and translated as a single protein [28]. The presence of arginine (residue 28) two amino acids upstream, indicates that the cleavage site for the removal of the targeting sequence lies between Thr29 and Cys30, a feature shared by cleavage sites in other mitochondrial proteins [29]. However, it is not known whether further processing occurs to generate the short form *in vivo*, or whether it arises through inappropriate proteolysis of the larger form during purification. Further work has demonstrated that peptidylprolyl *cis-trans*-isomerase (PPIase) activity is found in both the matrix (21 kDa) and intermembrane space (18 kDa) compartments of mitochondria [26]. But again it is unclear whether the PPIase recovered in the intermembrane space fraction is derived from CyP-D or is of a nonmitochondrial origin.

The present study has addressed these questions by importing [ $^{35}$ S]-precursor CyP-D (pCyP-D) into heart mitochondria and following its fate. It is concluded that pCyP-D is cleaved to its mature form in a single step and resides solely in the matrix compartment.

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**Abbreviations:** ANT, adenine nucleotide translocase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CSA, cyclosporin A; CyP, cyclophilin; PPIase, peptidylprolyl *cis-trans*-isomerase; PT, permeability transition; VDAC, voltage-dependent anion channel.

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## MATERIALS AND METHODS

### Materials

Primers and deoxynucleotide triphosphates were obtained from Pharmacia Biotech (St Albans, UK). PCR reaction buffer and *Pfu* DNA polymerase were supplied by Stratagene (Cambridge, UK). DNA purification was carried out using the GeneClean II Kit supplied by BIO 101, Inc (Vista, CA). [ $^{35}$ S]Methionine and X-Omat film were obtained from Amersham International (Amersham, UK). The TNT® T7 quick-coupled transcription/translation kits were obtained from Promega (Southampton, UK). Pre-stained SDS/PAGE standards were purchased from BioRad (Hercules, CA) and Protran BA85 nitrocellulose membranes were supplied by Schleicher & Schuell (Dassel, Germany). All other chemicals were obtained from Sigma (Poole, UK).

### Amplification of cyclophilin DNA template

Initial reactions amplified a cloned CyP-D cDNA, which was 39 bases short (5'-end) of the full-length sequence [24]. Primers were designed from the published 5'-end [28]. Precursor CyP-D template was amplified in two stages. The first step used the upstream primer (MTS-1) 5'-GACTCACTA-TAGGGCGAATTTCGCCATGGTAGCTCTGCGCTGCGGTCC-CCGCTGCTCGGTCTGCTCTCCGGCCCCGCGCTCCGCG-3' and the downstream primer (CyPD2) 5'-TGACTGGAATTC-TGTGACTTAGCTCAACTGGC-3'. The amplified product was purified and used as template for a second reaction using the primer (CyPD1) 5'-TGACTGTAATACGACTCACTA-TAGGGCGAATT-3' with primer CyPD2. Mature CyP-D template was amplified in a single reaction with the upstream primer (CyPD3) 5'-ACTGGGATCCTAATACGACTCACTATAG-GCGAGACCACCATGTGCAGCGACGCGCGAGCCCCGA-3' and primer CyPD2. The reaction conditions used throughout were denaturation at 94 °C (1 min), annealing at 45 °C (1 min) and polymerization at 72 °C (1.5 min) and 30 cycles (Personal Cycler; Biometra Germany).

### In-vitro transcription/translation of CyP-D

Coupled transcription/translation using the TNT® T7 quick-coupled kit was carried out according to the manufacturer's instructions using [ $^{35}$ S]methionine and 1 µg DNA template per 60-µl reaction. Reactions (30 °C, 90 min) were terminated by transfer to ice. Translated material was either used immediately or stored at -80 °C until required. Modifications are indicated in the text and figure captions.

### Preparation of import competent rat heart mitochondria

Mitochondria were prepared from the hearts of male Sprague-Dawley rats (200–250 g body weight). Hearts were removed, washed with ice-cold MSTEB buffer (210 mM mannitol, 70 mM sucrose, 10 mM Tris/HCl pH 7.2, 1 mM EGTA and 0.5 mg·mL<sup>-1</sup> fatty-acid-free BSA) and homogenized (Polytron Homogenizer; Kinematica, Lucerne) in the same buffer (50 mL·heart<sup>-1</sup>). After centrifugation (450 g, 5 min) two-thirds of the supernatant was decanted to fresh, chilled tubes and the mitochondria isolated by further centrifugation (5800 g, 10 min). The mitochondrial pellet was carefully re-suspended in cold MST (mannitol 210 mM, sucrose 70 mM and Tris/HCl 10 mM pH 7.2) and the previous centrifugation steps repeated.

Further washing did not improve the efficiency of import. Where required, mitochondria were ruptured by freeze-fracture. Briefly, mitochondria at a concentration of 10 µg·mL<sup>-1</sup> in MST buffer were frozen (liquid N<sub>2</sub>) and thawed five times. Aliquots were stored at -20 °C.

### Import of cyclophilin into isolated heart mitochondria

Freshly prepared heart mitochondria were diluted (2 mg·mL<sup>-1</sup>) in MRM buffer (250 mM sucrose, 10 mM Hepes, pH 7.5, 1 mM ATP, 5 mM sodium succinate, 0.8 mM ADP and 2 mM K<sub>2</sub>HPO<sub>4</sub>). The suspension was further diluted with an equal volume of KMH buffer (10 mM Hepes, 80 mM KCl, 2 mM Mg acetate, pH 7.5). Where required, mitochondria were de-energized prior to import by the addition of 1 µM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). Import reactions were initiated by the addition of 5 µL translated precursor to 200 µL heart mitochondria and conducted at 30 °C in an uncapped tube. Reactions were terminated after 40 min by transfer to ice and incubates split into two equal aliquots. One was treated with proteinase K (100 µg·mL). After 30 mins on ice, 2 mM PCH<sub>2</sub>SO<sub>4</sub>F was added to inhibit the protease and both samples diluted with 10 vol. of MRM buffer. Mitochondria were reisolated by centrifugation and washed with fresh MRM buffer without disturbing the pellet. Samples were dissolved in sample loading buffer for SDS/PAGE or used immediately in further experiments.

### Digitonin fractionation of heart mitochondria

Following import and proteinase K treatment, mitochondrial samples (200 µg) were diluted with 3 mg of freshly prepared mitochondria. Digitonin fractionation was then carried out as described previously [26]. Supernatants of pellets were assayed for [ $^{35}$ S], adenylate kinase and malate dehydrogenase [30].

Gel fractionation was carried out on selected samples of supernatants and pellets. Pellet samples were dissolved in 6% Chaps in MST. Samples were separated on a Superdex 75 column (Pharmacia Biotech) equilibrated with 150 mM NaCl/10 mM Hepes pH 7.5/0.5 mM EDTA/2 mM PCH<sub>2</sub>SO<sub>4</sub>F/leupeptin, antipain and pepstatin A (all 0.5 µg·mL<sup>-1</sup>). The [ $^{35}$ S]-containing fractions containing CyP-D were measured.

### Analysis of radiolabelled cyclophilin proteins

Translated proteins were analysed by SDS/PAGE [31]. Proteins were separated on 15% gels and transferred to nitrocellulose membranes at 100 V for 2 h in 48 mM Tris, 39 mM glycine, pH 9.2, methanol (20% v/v). Radiolabelled proteins were visualized by autoradiography.

## RESULTS

### Construction of CyP-D cDNA templates and expression of [ $^{35}$ S]-labelled CyP-D

Conventionally, mitochondrial precursors are derived by *in-vitro* transcription and translation from plasmid-borne coding sequences [32]. We have developed a similar approach using the PCR to create two cDNA templates coding for CyP-D. The first template contains the entire CyP-D reading-frame and codes for the mitochondrial precursor (pCyP-D). The second lacks the targeting region and codes for the mature protein (mCyP-D). Figure 1 shows schematically the design of both constructs and the position of amplifying primers.

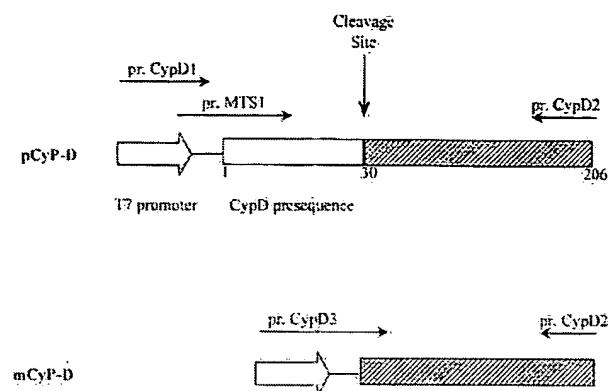


Fig. 1. Comparison of cyclophilin amplification products. The schematic compares the predicted amplified DNA products of precursor CyP-D (pCyP-D) and mature CyP-D (mCyP-D). Arrows indicate the position of primer binding and direction of amplification. The numbers below pCyP-D represent amino acid positions.

Rat CyP-D, including the N-terminal targeting sequence contains 206 amino acids [28]. N-terminal sequencing of CyP-D isolated from rat liver mitochondria revealed major and minor forms with residues 30 and 40, respectively, at the N-terminal end. Residues 1–29 of the nascent protein therefore form the mitochondrial targeting sequence, which is cleaved after import to yield mCyP-D (above). The possibility of a second downstream cleavage site at residue 39 is investigated in this study. For PCR, the template was a cloned rat CyP-D cDNA truncated (38 bases) at the 5'-end [24]. Codons for the targeting sequence were introduced by amplifying with an extended upstream primer (> 50 bp in length) [33]. Two amplification steps were required to add 84 bp to the original sequence. The mCyP-D template was amplified in a single step, adding a methionine codon ahead of the mature coding sequence (encoding residues 30–206). In addition, the upstream primer of both templates added T7 promoter sites ahead of the coding sequences to direct transcription. Sites surrounding the first methionine codon were modified to form Kozak consensus sequences (ACCATG) to enhance translation initiation [34].

Plasmid cDNA-directed coupled transcription/translation is sensitive to variations in  $Mg^{2+}$  concentration [35]. This is also the case for PCR-cDNA-directed transcription/translation. Figure 2A shows that efficient translation of [ $^{35}S$ ]-labelled pCyP-D was critically dependent on  $Mg^{2+}$ . Good expression was obtained with 3–5 mM  $Mg^{2+}$ , but higher or lower  $Mg^{2+}$  yielded negligible expression. However, this concentration optimizes translation at the expense of transcription [35]. We have compensated for this by maximizing the amount of

template DNA added to translation reactions that typically gave a final concentration of  $20 \mu\text{g}\cdot\text{ml}^{-1}$ . Again, this is similar to the optimum levels found for plasmid-based coupled transcription/translation systems.

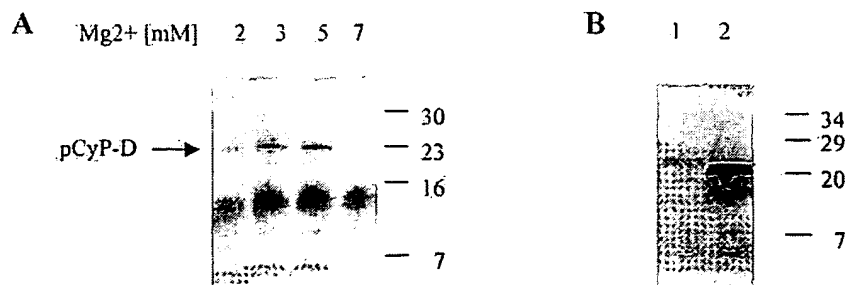
Figure 2B compares the products of *in-vitro* transcription/translation reactions from cDNA coding for pCyP-D and mCyP-D. In both cases, a single predominant band was generated. Under identical translation conditions, the level of expression of pCyP-D was consistently much lower than that of mCyP-D. Figure 2B shows a typical result, comparing equal volumes of translation reaction for each protein. Attempts to improve the expression of pCyP-D by directing translation from a plasmid cDNA under the control of T7 or T3 promoters did not improve the yield of this protein (data not shown). Bergsma and co-workers [6] noted that the cloned form of human mitochondrial cyclophilin did not express when cloned into *Escherichia coli*, but that this could be reversed by reducing the GC content of the N-terminal coding sequence. The GC content within the rat targeting sequence is 74% and the absence of this sequence from the cDNA coding for mCyP-D which expresses well, suggests that it is this region which inhibits efficient translation.

#### Radiolabelled pCyP-D is imported into mitochondria and processed to a 21-kDa form

We and others have reported that two forms of CyP-D are routinely purified from rat liver mitochondria [7,26]. N-terminal sequencing indicates that the short form is derived by N-terminal cleavage of the longer form [7]. As shown in Fig. 3, the two forms can also be purified from heart mitochondria. The two forms are clearly distinguishable, migrating at 21 kDa (lane 3) and 18 kDa (lane 4). As expected, CyP-D(21) comigrated with *in vitro* translated [ $^{35}S$ ]-mCyP-D (lane 2). Thus [ $^{35}S$ ]-mCyP-D is identifiable as one of the forms occurring naturally.

Confirmation that mCyP-D/CyP-D(21) represent the major CyP-D form *in vivo* was demonstrated by import of *in vitro*-translated pCyP-D into heart mitochondria (Fig. 4). Mitochondrial precursors, *in vitro*, are generated in the cytoplasm and imported post-translationally [36]. The targeting sequence allows specific binding to protein receptors in the outer membrane before import. True import, rather than superficial binding, was checked using two well established criteria for import [11], namely resistance to external protease and dependence of import on a high inner membrane potential. Two typical examples of import reactions are reported in Fig. 4, upper and lower panels. In the presence of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) to collapse the inner membrane potential and prevent import, precursor CyP-D bound to mitochondria (lanes 1) but was completely cleaved

Fig. 2. Expression of precursor and mature forms of cyclophilin D. (A) Expression of pCyP-D was enhanced by the addition of magnesium acetate to the concentrations indicated. (B) Comparable aliquots of translation reactions of pCyP-D (lane 1) and mCyP-D (lane 2) translated in the presence of 3 mM magnesium acetate are shown. Protein samples were separated by SDS/PAGE alongside prestained molecular mass markers (7–30 kDa), transferred to nitrocellulose and visualized by autoradiography.



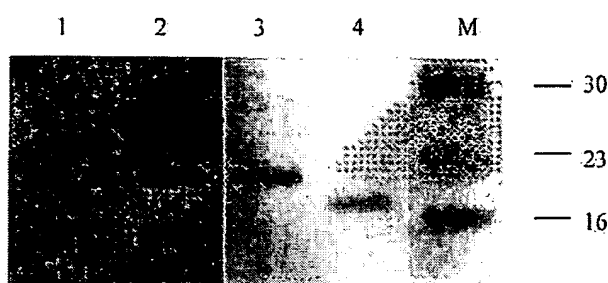


Fig. 3. Radiolabelled mCyP-D migrates with isolated CyP-D(21). Comparison of *in-vitro* expressed pCyP-D (lane 1) and mCyP-D (lane 2) with CyP-D(21) (lane 3) and CyP-D(18) (lane 4) purified directly from rat heart mitochondria. Methods were as in Fig. 2. Purified proteins (lanes 3 and 4) and markers were visualized by staining the membrane with ponceau red. The amounts of pCyP-D and mCyP-D have been adjusted to give equal band intensity.

by exogenous proteinase K (lanes 2). In some experiments (e.g. lane 1, upper panel), but not all (e.g. lane 1, lower panel), some cleavage of bound pCyP-D occurred in the presence of CCCP. However, this protein did not persist following proteinase K treatment and was evidently not imported. In experiments showing cleavage of bound CyP-D (lane 1, upper panel) we also observed a small proportion (3–5%) of mitochondrial malate dehydrogenase in the supernatant fraction. In experiments in which cleavage was not observed in the presence of CCCP (e.g. lane 1, lower panel) freezing and thawing of the mitochondrial preparation did lead to cleavage of pCyP-D to produce the second band (in the presence of CCCP). Taken as a whole, these data indicate that when cleavage was observed in CCCP-treated mitochondria, it was due to a small amount of mitochondrial lysis and release of processing peptidase.

In the absence of CCCP, i.e. in the energized state, pCyP-D again bound to heart mitochondria (lanes 3, upper and lower panels) and produced a single lower band. From densitometric

analysis, the lower bands represented 45% and 38% of the radiolabelled protein. The lower band was clearly resistant to proteinase K (lanes 4) and co-migrated with [ $^{35}$ S]mCyP-D (lanes 5) and therefore this *in-vitro* generated protein is the same as CyP-D(21) isolated from mitochondria.

Import was also attempted in rat liver mitochondria. However, residual free [ $^{35}$ S]methionine, introduced into the import reaction from the translated sample, became incorporated into intramitochondrial proteins, giving rise to multiple radiolabelled proteins that were resistant to protease treatment. Attempts to block synthesis with translation inhibitors were only partially successful. The absence of this problem in mitochondria derived from heart tissue made them the preparation of choice.

From Fig. 4 (upper and lower panels) it is evident that pCyP-D was processed to a single intramitochondrial species. A further example of this single intramitochondrial band (on SDS/PAGE) following import, is given in Fig. 6B (inset). In some experiments (e.g. Fig. 4, lower panel, lane 4), but not others (e.g. Fig. 4, upper panel; Fig. 6B inset), some 'fraying' of the lower edge of the 21 kDa band was seen after proteinase K treatment. This evidently resulted from the added proteinase K, rather than any endogenous peptidase, since it was not seen in the imported band before proteinase K treatment (Fig. 4, lower panel, lane 3). These results are typical of six experiments with heart mitochondria. In none of these experiments was a smaller band observed, corresponding to CyP-D(18). Moreover, no smaller band arose when mitochondria were maintained for several hours after import (data not shown). CyP-D is functionally associated with the PT pore (Introduction). We have also tested whether conditions that lead to pore activation (100  $\mu$ M  $\text{Ca}^{2+}$ , 5 mM  $\text{P}_i$ , 500  $\mu$ M tertbutylhydroperoxide) produced a lower molecular mass species. However, none was found (data not shown).

In *Neurospora*, mitochondrial CyP is expressed as a 24 kDa precursor. After import, the precursor is cleaved in two steps via a 21 kDa intermediate to the 20 kDa mature

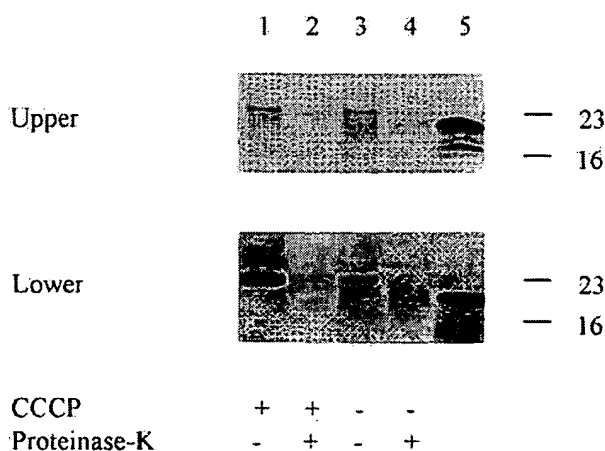


Fig. 4. Import of pCyP-D into rat heart mitochondria requires an inner membrane potential. Freshly prepared rat heart mitochondria were incubated with [ $^{35}$ S]-labelled pCyP-D in the presence (de-energized mitochondria, lanes 1 and 2) and in the absence of CCCP (energized, lanes 3 and 4). After import, samples (lanes 2 and 4) were treated with proteinase K. Radiolabelled proteins were analysed as in Fig. 2. A mCyP-D control is included (lanes 5) for comparison with import products. Upper and lower panels show experiments with different mitochondrial preparations.

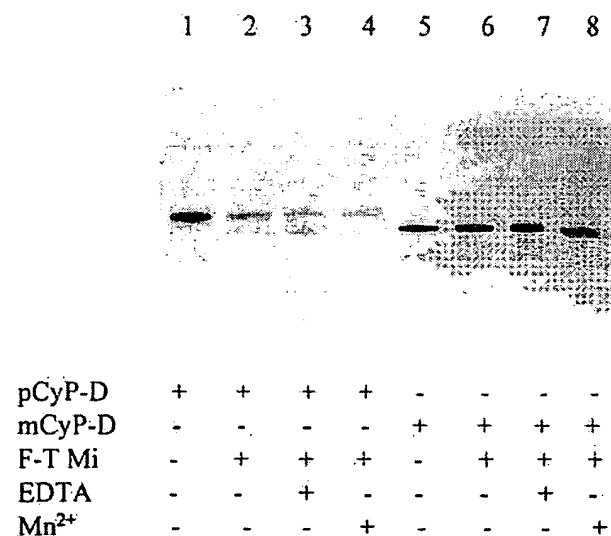
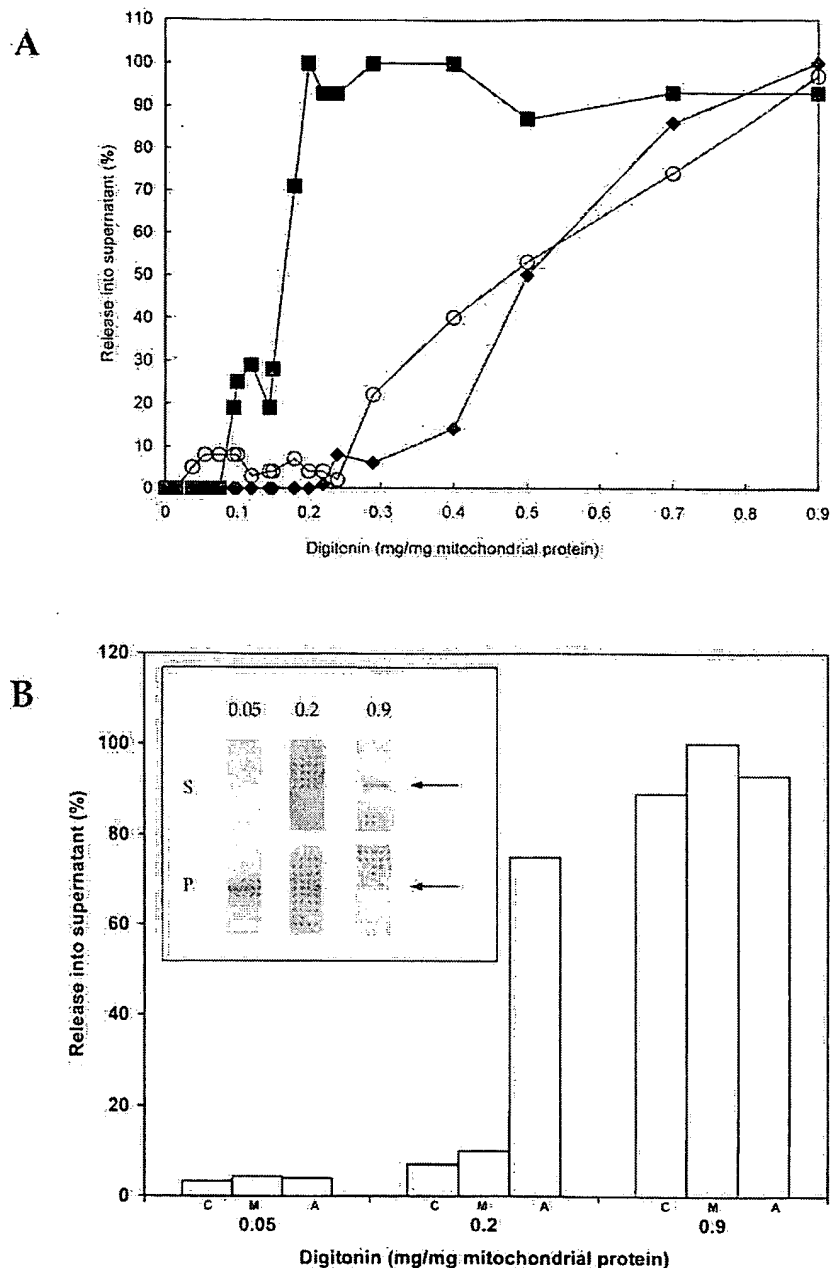


Fig. 5. Proteolysis of pCyP-D by ruptured mitochondria. pCyP (lanes 1–4) and mCyP-D (lanes 5–8) were either left untreated (lane 1 and 5) or treated with 10  $\mu$ g mitochondria ruptured by freeze-thaw (F-T Mi)  $\text{Mn}^{2+}$  (1 mM) and EDTA (1 mM) were added as indicated. Samples were incubated at 30  $^{\circ}\text{C}$  for 60 min before termination with SDS/PAGE loading buffer.



**Fig. 6. Intramitochondrial location of imported CyP-D.** Mitochondria were loaded with [<sup>35</sup>S]-CyP-D: (A) The release of [<sup>35</sup>S]-CyP-D (○), adenylate kinase (■) and malate dehydrogenase (◆) were measured at increasing digitonin concentrations. (B) [<sup>35</sup>S]-CyP-D release was quantified after isolation from the supernatant fraction by gel filtration (Materials and methods) and compared with the marker enzymes adenylate kinase (A) and malate dehydrogenase (M). Three digitonin concentrations were used. The inset shows samples of the supernatant (S) and pellet (P) analysed by SDS/PAGE and autoradiography. Arrows indicate the position of mature CyP-D.

form. The second step is catalysed by a Mn<sup>2+</sup>-dependent peptidase. Therefore we also tested for a similar final processing step in heart mitochondria by exposing mCyP-D and pCyP-D to ruptured mitochondria in the presence and absence of Mn<sup>2+</sup> (Fig. 5). mCyP-D (lane 6) resisted further digestion irrespective of the presence of Mn<sup>2+</sup> or EDTA to chelate endogenous divalent cations (lanes 7 and 8). Under similar conditions pCyP-D was processed to mCyP-D, but no further (lanes 1–4). Thus again, we obtained no evidence for a second, Mn<sup>2+</sup>-dependent processing step in rat heart mitochondria.

#### Mitochondrial sublocation of [<sup>35</sup>S]-labelled CyP-D

On subfractionation of liver mitochondria, cyclophilins are recovered in both matrix (70–80% of total) and intermembrane

space (20–30%) compartments [26]. Nicolli *et al.* [37] have drawn attention to the contamination of conventionally prepared mitochondria with non-mitochondrial cyclophilin, i.e. CyP-A (cytosol) and CyP-B (ER). The successful import and processing of CyP-D in radiolabelled form (Fig. 6) allowed us to determine its submitochondrial location precisely using the digitonin fractionation procedure. Digitonin lyses membranes through interaction with cholesterol. The higher cholesterol content of the outer membrane (0.06 mmol·mg<sup>-1</sup>) compared with the inner membrane (0.02 mmol·mg<sup>-1</sup>) makes this more susceptible to lysis at lower digitonin concentrations [38]. Fractionation was quantified from the release of marker enzymes for the intermembrane space (adenylate kinase) and the matrix (malate dehydrogenase).

Figure 6A shows that the profile of [<sup>35</sup>S] release with increasing digitonin concentration mirrored that of the matrix

marker, malate dehydrogenase, and not adenylate kinase. The best separation of the matrix and intermembrane space fractions occurred at about 0.2 mg digitonin-mg mitochondrial protein<sup>-1</sup> (Fig. 6A). In three separate experiments the amount of [<sup>35</sup>S] release into the supernatant at 0.2 mg digitonin-mg protein<sup>-1</sup> ( $5 \pm 2\%$  mean  $\pm$  SEM) was the same as malate dehydrogenase ( $6 \pm 3\%$ ), and quite unlike adenylate kinase ( $85 \pm 5\%$ ). These data indicate that CyP-D(21) is restricted in the matrix space.

To check this, supernatant and pellet samples obtained at three concentrations of digitonin were fractionated by gel filtration and the radiolabel in the CyP-D peaks was quantified (Fig. 6B). Again, the CyP-D distribution accurately reflected that of malate dehydrogenase and not adenylate kinase. In addition, samples of both supernatant and pellet at these digitonin concentrations were analysed by SDS/PAGE (Fig. 6B, inset). Transfer of CyP-D from the pellet (i.e. intact mitochondria or mitoplasts) to the supernatant only occurred at the highest concentration of digitonin. Within the limits of detection therefore, these data confirm that CyP-D is located entirely within the matrix.

## CONCLUSIONS

The present data demonstrate that PCR-derived cDNAs can form efficient templates for mitochondrial precursor proteins. Promoter sequences and modifications to the flanking sequences can be introduced through amplification with extended primers ( $> 50$  bp). The factors which influence transcription and translation from PCR-derived templates are similar to those observed from plasmid-derived cDNAs [35]. Furthermore, PCR-derived templates do not require purification or restriction endonuclease digestion before use in translation reactions. This dramatically reduces the time required to generate translated proteins.

We have used this technique to generate the mitochondrial precursor and mature form of rat CyP-D. The precursor was successfully imported into rat heart mitochondria and processed to a single 21 kDa protein. It has been proposed that multiple processing of CyP-D occurs and generates two matrix cyclophilins [7]. However, we have been unable to demonstrate multiple cleavage of the precursor either following import or under a variety of conditions in which mitochondrial proteases are released. We conclude that a single form of CyP-D exists in the matrix, at least in heart mitochondria. In fungi, two ways of processing mitochondrial cyclophilins have been identified. In yeast, mitochondrial CyP is processed in a single step after import, whereas in *Neurospora crassa*, imported CyP is cleaved twice to produce the final mature form. From the present study, mammalian mitochondrial CyP seems to be processed as in yeast rather than as in *Neurospora*.

Cyclophilins and their homologues have been identified in most cellular locations including the cytoplasm [3,39], ER [40,41] and nucleus [42] as well as the mitochondrial matrix (this study). Their general roles have yet to be established, but in view of their ability to catalyse rotation of prolyl peptide bonds (Introduction), a function in protein folding seems likely. Certain observations support this role. Folding of transferrin in the ER is inhibited by CSA consistent with catalysis by CyP-B [9]. Yeast mitochondria deficient in functional mitochondrial (matrix) CyP show impaired folding of imported proteins [10]. Matrix CyP-D (this study) may perform a similar role. Along these lines, it might be anticipated that the intermembrane space would also contain a cyclophilin as well as the mitochondrial matrix (this study), to catalyse folding of

proteins newly imported into that compartment. From the present study, however, any intermembrane space CyP is unlikely to be derived from CyP-D.

In addition CyP-D is a component of the PT pore which is believed to be involved in both necrotic and apoptotic cell death (Introduction). CSA blocks the PT pore [12] by interacting with CyP-D [24, 25 and references therein]. However, the role played by CyP-D in the PT pore has not been resolved. Kroemer and co-workers [43] suggested that it may be involved in the recruitment of the proapoptotic protein Bax. We have shown that a recombinant glutathione S-transferase (GST)-CyP-D fusion binds strongly to complexes of VDAC and ANT which form the PT pore, and that CSA binding to CyP-D within the complex produces pore blockade [24]. Evidently, CyP-D is an integral component of the PT pore complex, rather than loosely associated. The present study has shown that CyP-D is restricted to the matrix compartment. Therefore, CyP-D must interact with ANT in the inner membrane, rather than with VDAC in the outer membrane, and must bind to ANT from the matrix side of the inner membrane. This is in line with studies elsewhere demonstrating interaction between CyP-D and ANT alone [25]. Cross-linking of imported radiolabelled CyP-D may allow the nature of this interaction to be elucidated, and this is currently under way in this laboratory.

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